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(54) Title: BRAIN PROTEIN MARKERS

(57) Abstract: Protein markers for various psychiatric and neurological disorders were discovered. Diagnostic assays for these conditions were based on the levels of individual or combinations. The markers are targets or drug discovery for therapeutic and prophylactic uses.

## BRAIN PROTEIN MARKERS

### FIELD OF THE INVENTION

5

The instant invention relates to proteins found in the brain, which are markers for neurological and psychiatric disorders, and their use for diagnostic, therapeutic, prophylactic and drug discovery purposes.

10

### BACKGROUND OF THE INVENTION

Serious psychiatric disorders such as schizophrenia, bipolar disorder and major depressive disorder, are brain diseases of unknown origin which are major causes of morbidity throughout the world. Epidemiological and pathological studies indicate a role for both genetic and environmental factors in the etiology and pathogenesis of such disorders. However, specific disease-associated genes or pathogenic agents have not as yet been identified. There are, at present, no biological or pathological markers that are highly associated with specific psychiatric disorders.

25

Two-dimensional electrophoresis is an analytic method that can quantify the levels of individual proteins of complex biological samples (1) and can allow comparison between diagnostic groups. The technique has been applied to the limited study of brain tissues (2, 3) and cerebrospinal fluid (4, 5,

30

6, 7) obtained from individuals. Recent advances in image analysis, sequencing and other proteomic techniques allow for more detailed analysis of protein expression in biological samples (8, 9). In spite of those and other great efforts, no biological marker has been documented at the pathological, cellular or molecular level, suggesting that a number of complex but subtle changes underlie those illnesses.

10

#### SUMMARY OF THE INVENTION

An object of the instant invention is to determine which proteins or combinations of proteins constitute markers for brain disorders.

It is another object of the instant invention to diagnose brain disorders by measuring those markers in a biological sample.

It is still another object of the instant invention to use the markers as targets for developing agents interacting with those markers, such as agonists or antagonists, to develop treatments, prophylaxis or disease models.

It is a further object to alter the levels of those proteins by altering gene expression, protein processing and protein degradation.

It is yet another object to determine altered metabolic pathways and to develop bio-affecting agents that ameliorate the disorder by affecting other proteins in the pathway.

An additional object is to determine markers for other brain disorders that have a component affecting those brain protein markers.

It is still a further object to develop and use  
5 binding assays and functional assays to measure the markers individually or in specified combinations.

Thus, in the instant invention, proteomic technology was used to survey postmortem brain tissue to identify changes linked to the various  
10 diseases. Two-dimensional gel electrophoresis and mass spectrometric sequencing of proteins allowed the comparison of subsets of expressed proteins among a large number of samples. That analysis was combined with a multivariate statistical model to  
15 study changes in protein levels.

For example, eight protein markers display disease-specific alterations. Six showed decreased expression compared with controls for one or more diseases. Four of those are forms of glial  
20 fibrillary acidic protein (GFAP), one is dihydropyrimidinase-related protein 2, and the sixth is ubiquinone cytochrome C reductase core protein 1. Two proteins, carbonic anhydrase I and fructose biphosphate aldolase C, are increased in one or more  
25 diseases as compared to controls.

Additionally, sets of certain of the 19 markers are diagnostic when used in particular combinations.

From those markers, one may identify novel pathogenic mechanisms of human neurological and  
30 psychiatric diseases.

## BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a depiction of a master pattern of  
5 2-D gels.

Figure 2 is a depiction of a 2-D gel with an  
insert depicting certain protein markers in abnormal  
amounts.

Figure 3 is a plot of protein levels in various  
10 diseases.

Figure 4 is a graph comparing normal and  
depression brain levels of certain combinations of  
protein markers.

Figure 5A depicts a master pattern of 2-D gels  
15 with the 19 spots located.

Figure 5B depicts only the 19 spots from Figure  
5a.

Figure 6 provides graphs of the levels of each  
spot with the normal group presented first followed  
20 by the depression group.

Figure 7 is a table of the 19 spots and the  
coefficients to multiply each value to obtain the X  
and Y coordinate numbers of Figure 4.

## 25 DESCRIPTION OF THE PREFERRED EMBODIMENTS

While not wishing to be bound by any particular  
theory, applicants believe the mechanisms of action  
and prior discoveries are correct. For  
30 understanding of the instant invention, certain

mechanisms are set forth which may not be exactly correct with better understanding of the brain.

The term "brain disorders" includes a large number of psychiatric and neurological conditions which generally are considered undesirable, such as schizophrenia, bipolar disorder (manic/depression), major depressive disorder, dementia, infectious and degenerative conditions resulting in loss of brain function, psychiatric conditions (as defined by the DSM) and neurological disorders due to central nervous system disorders.

A "biological sample" includes any body fluid or tissue from an animal containing a protein marker and/or nucleic acid(s) encoding the marker and/or substrates for the protein and/or products produced by the marker.

"Subject" is an individual person or animal that is suspected of having a brain disorder, is a test individual or a control.

The instant invention resulted from discoveries made with a large number of post-mortem brain samples collected from well-characterized individuals with schizophrenia, bipolar disorder, and major depressive disorder as well as equivalent samples from matched, unaffected controls.

Two-dimensional gel electrophoresis was employed, followed by sequencing by electrospray mass spectroscopy, to identify proteins that show varied levels in the frontal cortex of individuals with defined psychiatric disorders. The relationship of those proteins to clinical diagnosis and other

clinical and demographic parameters was evaluated further by application of a multivariate statistical model.

The conditions used enabled a comparison and  
5 evaluation of the subset of proteins that were highly abundant, relatively soluble proteins with pIs between 4-7. The methods resulted in the identification of 8 disease-related changes in protein levels in the frontal cortex region of  
10 individuals with serious psychiatric diseases. Different methods of analysis identified a combination of 19 different proteins that correlated to serious psychiatric disease.

Eight protein species displayed differences in  
15 levels that were associated strongly with at least one clinical diagnosis. The relationship between the statistically significant changes and disease were revealed by using large sample sets combined with multivariate analysis to account for individual  
20 variation and a number of confounding antemortem and postmortem factors. The conditions used to run, stain and analyze the gels were adjusted to study a small subset of all available proteins, namely the highly abundant, soluble proteins with pIs between  
25 4-7.

Despite that limitation, the identification of the proteins provides information on the possible pathways or processes involved in the diseases. For example, there was a decrease in the expression of  
30 dihydropyrimidinase-related-protein 2 (DRP-2) in the brains of individuals with schizophrenia, bipolar



disorder and major depressive disorder. The animal homologs of DRP-2 (rat CRMP-62, mouse unc-33, chick TOAD-64) are brain specific enzymes that play a crucial role in development (12-15). In humans, an  
5 absence of dihydropyrimidinase is observed in conjunction with severe neurological impairments occurring through delayed or arrested development, and neuronal degeneration with secondary delay of myelination (16, 17). Altered levels of DRP-2  
10 protein are thus consistent with alterations in brain development, which may play a role in a wide range of neurological and psychiatric disorders.

There was a significant increase in the level of carbonic anhydrase I in the brains of individuals  
15 with depression. Carbonic anhydrase I is the erythrocyte form of the enzyme, while the isoform, carbonic anhydrase II, is expressed in glial cells, myelin and choroid plexus (18-20) and is also one of the principal determinants of pH fluxes within  
20 neural cells (21). That observation confirms the report of Hayes (18) who found that treatment with the carbonic anhydrase inhibitor, acetazolamide, resulted in a significant improvement in symptoms in individuals in the depressive phase of bipolar  
25 disorder. Levels of the protein markers including carbonic anhydrase may be determined in other brain areas to determine the utility of therapeutic interventions directed at altering the levels of activity of that enzyme.

30 Increased levels of brain-specific fructose-biphosphate aldolase C were found in

samples obtained from individuals with schizophrenia, bipolar disorder and unipolar depression. (Mass spectrometry sequencing of the same protein spot revealed peptides identified as aspartate aminotransferase. That protein is not  
5 expressed in brain (27) and thus was considered a likely contaminant in the preparation.)

Aldolase immunoreactivity was elevated in CSF (22); the activity was increased in the serum of  
10 psychiatric patients in a clinical state-dependent manner (23-26) and also was abnormal in the first-degree relatives thereof (26). Clearly, fructose-biphosphate aldolase C has a role in brain metabolism and will be a focus of drug development.

15 There was a statistically significant decrease in the levels of the core protein 1 of the mitochondrial ubiquinone cytochrome C reductase complex in the brains of individuals with depression. Subtractive cDNA libraries (28)  
20 revealed an excess of mitochondrial message in the brains of some individuals with schizophrenia. That mitochondrial enzyme also provides a diagnostic and drug discovery target for psychiatric disorders.

Four of the eight protein species with  
25 disease-specific differences of expression were identified as isoforms of astrocytic glial fibrillary acidic protein (GFAP). The finding of multiple species of GFAP with different isoelectric points but similar molecular weights is probably a  
30 reflection of the presence of different levels of post-translational modification of that brain

protein. Other researchers also have identified modified forms of GFAP using two-dimensional electrophoretic methods (2, 29, 30 and 31).

The level of GFAP expression is modulated by many factors, including cytokines, hormones and growth factors, throughout brain development (32, 33). GFAP can be phosphorylated at five sites (Thr7, Ser8, Ser13, Ser17 and Ser38), often in response to numerous stimuli (34). Phosphorylation results in the disaggregation of GFAP molecules (35) and results in a protein molecule with a greater negative charge (36). Spots 311 and 500 may represent phosphorylated forms of the protein. Alternatively, the species may represent other post-translational modifications resulting in, for example, acetylation, glycosylation or deimination (35), which also would generate molecules which migrate in a more acidic fashion on two-dimensional gels.

Several studies have compared GFAP levels in the brains of individuals with schizophrenia and unaffected individuals. No significant disease-related alterations in GFAP have been detected by in situ immunohistochemistry or by one-dimensional immunoblotting methodologies (37). Additionally, those methods would not be likely to detect differences related to phosphorylation or other post-translational modifications detectable by two-dimensional electrophoresis. Comings et al. 1982, employing two-dimensional electrophoretic methods, found absence of some charge variant forms

of GFAP in a small group of individuals with schizophrenia and depression (~67% of analyzed samples) as compared to unaffected controls and other non-psychiatric neurological diseases (0-28%).

5        It is not known if the alterations in the levels of the GFAP isoforms reflect disease-specific alterations in pathways controlling post-translational modification. GFAP decreases have been documented in Down's Syndrome (38) and in  
10       response to tumor necrosis factor  $\alpha$ , basic fibroblast growth factor and glucocorticoids in cell cultures (32, 39). GFAP is known to increase dramatically in response to acute infection or neurodegeneration (40). However, GFAP levels can be  
15       decreased in response to chronic infection with viruses such as pseudorabies (41), varicella zoster (42) and HIV-1 (43). Of particular interest in the case of HIV-1 is the finding that the viral envelope glycoprotein gp120 can inhibit directly the  
20       phosphorylation of GFAP (44).

      It is of note that some of the alterations were found in individuals with different psychiatric diagnoses and may represent features that are common to the different diseases, such as non-specific  
25       markers of inflammation or a common second messenger pathway. The analyses of brain proteins by proteomic methods identify disease-related changes in brain proteins and directs discovery of novel pathways for the diagnosis and treatment of serious  
30       human psychiatric diseases.

TABLE 1. Eight spots significantly affected by one or more diseases

Protein ID#	Change Compared to Controls	Diseases Affected (Significance in Multivariate Analysis)	Other variables with Significant effect at $p < 0.01$ (direction)
74	Decrease	Depression (p=0.015)	None
76	Decrease	Depression (p=0.0089)	CO Poisoning (decrease) Freezer Interval (decrease)
119	Decrease	Depression (p=0.0040)	Freezer Interval (decrease)
141	Decrease	Schizophrenia (p=0.01) Bipolar Disorder (p=0.014) Depression (p=0.0005)	Other Suicide (increase)
182	Increase	Schizophrenia (p=0.0023) Bipolar Disorder (p=0.0061) Depression (p=0.0009)	Alcohol abuse (decrease)
226	Increase	Depression (p=0.0029)	None
311	Decrease	Schizophrenia (p=0.0001) Depression (p=0.0020)	CO Poisoning (decrease) Freezer Interval (decrease)
500	Decrease	Schizophrenia (p=0.0011) Bipolar Disorder (p=0.0042)	Freezer Interval (decrease)

**TABLE 2. ID of Differentially Expressed Proteins**

ID#	Disease Related Change	Name* (Ascension #)	Peptides sequenced
74	Decreased in depression	Ubiquinol cytochrome C reductase complex core Protein 1 (P31930)	LSRADLTEYLSTH YK, NNGAGYFLEHLAFK
76	Decreased in depression	Glial fibrillary acidic protein (P14136)	ALAAELNQLRAK
119	Decreased in depression	Glial fibrillary acidic protein (P14136)	FADLTDAAR, LEAENLAAYR
141	Decreased in schizophrenia, bipolar disorder and depression	Dihydropyrimidinase related protein 2 (D78013)	VENLYPR, MDENQFVAVTSTNA AK, MVIPGGIDVHTR
182	Increased in schizophrenia, bipolar disorder and depression	Fructose-biphosphate aldolase (P09972)	TQGLD, SALALLE**, EEEASFNLNAL NR**, QASAL
		Aspartate aminotransferase*** (P17174)	RFSLLGLLPLY EH***
226	Increased in depression	Carbonic anhydrase I (P00915)	LLSNVEGDNAVPM QH, IINVGHSHFVNFE, LYPIANGNNQS?V
311	Decreased in schizophrenia and depression	Glial fibrillary acidic protein (P14136)	PVQTFSN, QDLA, VDFSLAGA, PVQTF
500	Decreased in schizophrenia and bipolar disorder	Glial fibrillary acidic protein (P14136)	PVQTFS, AGAL

\*Protein sequenced by electrospray mass spectroscopy of tryptic peptides as described herein.

\*\*Second leucine in each of the sequences is an isoleucine in the identified protein.

\*\*\*Putative liver specific transcript, probably a contaminant<sup>27</sup>.

TABLE 3. Comparison of theoretical and measured  
GFAP values.

	GFAP species	PI	MW (Daltons)
Theoretical		5.42	49,880
Measured	76	5.40	48,700
	119	5.35	49,000
	189	5.28	50,000
	311	5.23	51,000
	500	5.20	52,000

5        Theoretical values were obtained by entering  
the amino acid sequence P14136 into the pI/MW  
calculator at <http://expasy.hcuge.ch/chzd/pi>  
tool/html.

10        The separated proteins can be isolated from the  
gels following electrophoresis. The individual  
spots can be excised and the proteins contained  
therein extracted. The extracted proteins can be  
employed for a variety of uses, such as, immunogens,  
if antibodies to those proteins already do not  
15        exist.

Hence, practicing known methods, the  
polypeptides are used to immunize a host. Adjuvants  
and carriers to enhance immunogenicity and  
reactivity can be used. The host animal is  
20        sensitized using a regimen as known in the art with  
suitable immunogen amounts, doses, number of doses,

site of administration and so on as known in the art. Following the immunization regimen, blood is drawn and the antiserum produced.

Alternatively the host animal, particularly if  
5 a mouse, can serve as a source of antibody-producing cells for the manufacture of monoclonal antibodies practicing known methods. Also, as known in the art, immunization of immune cells by protein can occur in vitro with the sensitized cells serving as  
10 the cells to be fused with myeloma cells. The resulting antibody, whether polyclonal or monoclonal, can be tested for specificity as known in the art. For example, if over expressed or underexpressed in brain tissue of sick individuals  
15 as provided herein, the brain tissue that was compared originally can be assayed using the antibody in an in situ technique, such as an immunohistochemical method. Thus, diseased and normal brain tissue is exposed to the antibody and  
20 then the tissue is exposed to a reporter molecule to reveal binding of the antibody. Alternatively, the primary antibody can be labeled with a reporter molecule. In the case where proteins are overexpressed in diseased tissue, greater antibody  
25 binding is observed as compared to controls. In the case of protein underexpression, lower levels of antibody binding are observed in diseased tissue as compared to controls.

Such antibody can serve as a diagnostic reagent  
30 in any of a variety of known assays, particularly if the protein of interest is found in a body fluid in



a diagnostic fashion. Suitable body fluids would be cerebrospinal fluid, serum, saliva, tears and so on. Otherwise, other sorts of known immune assays using any of a variety of samples, such as biopsies, can  
5 be employed.

For diagnostic uses, one may perform 2-dimesional gel electrophoresis as the key protein markers are determined. Simple enzyme assays, immunoassays or other binding assays for the key  
10 protein markers, however, may be preferred.

While the instant invention is best used for diagnostic purposes and for drug discovery, it also determines which proteins are in abnormally high and low levels in various brain disorders. When a  
15 protein is found in abnormally low levels, the protein may be administered, for example, parenterally or intrathecally to compensate for the insufficiency. Encapsulating the protein may be preferred to aid in intracellular delivery.  
20 Liposomes and micelles are conventional techniques for encapsulating proteins along with suitable pharmaceutically acceptable carriers.

As an alternative to providing protein replacement therapy, one may provide gene therapy by  
25 adding extra copies of the gene encoding the protein of interest. It may be regulated by natural regulatory sequences or by different regulatory sequences depending on the level of expression desired. The gene may be delivered by any of a  
30 number of gene therapy vectors, known per se. Alternatively, expression may be altered by inducing

a point mutation in a regulatory sequence to the gene encoding a brain protein marker, using techniques known per se.

When a protein is found in abnormally high  
5 levels, antisense nucleic acids may be administered to reduce expression by techniques known per se.

While the bio-regulating methods are generally used therapeutically, the methods also may be used as maintenance treatment or prevention for  
10 individuals having a genetic predisposition to a disorder or ones exposed to environmental conditions, medication or event (trauma, stress and loss) that may precipitate a disorder.

In the field of drug discovery, protein markers  
15 that are found at abnormal levels represent targets for the screening of small molecule compounds as well as specific binding elements. Agonists, antagonists and enzyme inactivating drugs represent many of the modern drugs. All of the brain protein  
20 markers of the instant invention determinable by 2-dimensional gel electrophoresis may be so used.

Furthermore, unlike other protein detection assays, 2-dimensional gel enables the simultaneous measurement of many proteins. That allows deducing  
25 metabolic pathways by determining a series of proteins with abnormal concentrations, the expression of which may be governed in a coordinated fashion. Other proteins in the metabolic pathway represent alternative targets for drug discovery.

30 The invention now will be described in the following non-limiting examples.

**EXAMPLE 1:        GENERATION OF PROTEIN MARKERS FROM  
BRAIN TISSUE**

Eighty-nine postmortem human brains obtained as  
5 part of the Stanley Foundation Brain Collection were  
collected from designated medical examiners with the  
permission of the families, processed and stored at  
-70°C. Two senior psychiatrists made diagnoses,  
using DSM-IV (Diagnostic and Statistical Manual of  
10 Mental Disorders, American Psychiatric Association,  
1994) criteria based on hospital records and  
telephone interviews with family members.

Brain tissue was analyzed from individuals with  
the following diseases: schizophrenia (N=24),  
15 bipolar disorder (N=23), major depressive disorder  
without psychotic features (N=19) and unaffected  
controls (N=23). Tissue used in the studies was  
obtained from the frontal cortex (Brodmann Area 10)  
of the cerebral hemisphere which had been frozen on  
20 collection.

Approximately 0.2 grams of frontal lobe tissue  
was homogenized in 8 volumes of solubilizing  
solution (2% NP-40 (Sigma), 9 M urea (Gibco), 0.5 M  
dithiothreitol, and 2% ampholytes (pH 8.0-10.5  
25 Pharmacia)) for 30 seconds using a Tissue Tearor at  
setting 2. The insoluble material was removed by  
ultracentrifugation at 100,000 rpm, 20° C, for  
30 minutes. Homogenates were stored at -80°C until  
use.

Proteins were resolved by 2-D gel electrophoresis using the 20 x 25-cm ISO-DALT® 2-D gel system operating with 20 gels per batch. All first dimension isoelectric focusing gels were prepared using the same batch of ampholytes (BDH 4-8A). Three microliters containing roughly 100 µg of solubilized brain protein were applied to each gel, and the gels run for 33,000 to 34,500 volt-hours using a progressively increasing voltage protocol implemented by a programmable high voltage power supply. The second dimension gels were prepared using an ANGELIQUE® computer controlled gradient casting system where the top 5% of the gel was 11% T acrylamide, and the lower 95% of the gel varied linearly from 11% to 18%. Second dimension slab gels were run in groups of 20 in thermostabilized (10° C) DALT tanks with buffer circulation.

Slab gels were fixed and stained for protein using a colloidal Coomassie Blue G-250 procedure. Staining proceeded for about 4 days to reach equilibrium intensity. Coomassie Blue was chosen as a reproducible, quantitative dye as compared to the equally sensitive fluorescent stains or the ten-fold more sensitive silver stain. Each slab gel was digitized in red light at 133 µm resolution, using an Eikonix 1412 scanner. Each gel then was processed using the KEPLER® software system, procedure PROC008b to yield a spot list giving position, shape and density information for each spot detected.

**EXAMPLE 2: DETERMINATION OF BRAIN PROTEIN  
MARKERS**

5           A master 2-D pattern of human frontal cortex  
was constructed using multiple 2-D images, which  
included the common polymorphisms and additional  
spots encountered in the full set of brain samples  
(Figure 1). All spots were identified with a  
10       number. The individual gels then were matched to  
the master in two steps. First, an experienced  
operator manually matched about 50 protein species  
with the corresponding spots on the master pattern,  
creating a set of reference points. Subsequently,  
15       an automated program identified all other protein  
species based on the relation to the manually  
matched spots. All spots were normalized by the sum  
of the densities of a small set of spots that were  
well resolved and present in almost all gels. A  
20       parallel set of gels was co-run with a rat liver  
protein preparation, and the proteins of the  
well-characterized rat liver proteome (10) were used  
as molecular weight and pI standards.

          The levels of individual protein species  
25       (measured as described above) were predicted by  
variables representing diagnostic, demographic,  
lifestyle, mode of death and subsequent tissue  
storage characteristics using a multivariate  
statistical model. Over 20 such variables are known  
30       for each of the samples. Because of strong  
correlations with one another (colinearity), as well

as a need to limit variables due to the small number of samples, the number used in the model was reduced specifically to avoid colinearity between the independent variables.

5       The final model contained the following 10 antemortem and postmortem dependent variables that could affect protein levels in brain tissue: age (years), alcohol use (scaled 0-5), drug abuse (scaled 0-5), death by carbon monoxide poisoning  
10 (yes/no), suicide other than carbon monoxide poisoning (Y/N); diagnosis (schizophrenic, bipolar, depressed or unaffected), body mass index ( $\text{kg}/\text{m}^2$ ), brain weight (grams), freezer storage interval (months) and pH of occipital lobe. Alcohol and drug  
15 abuse scales and the pH measurement methods have been described previously (11).

Proteins of interest were excised from the two-dimensional gels, digested with trypsin in situ and peptides were eluted and subjected to sequencing  
20 by electrospray mass spectrometry. The deduced peptide sequences were compared with the Swiss protein database for known matches.

A total of 217 spots were matched between all 89 gels. The proteins were quantified as described  
25 above. The resulting protein quantities then were analyzed in a multivariate model to eliminate the possible confounding effects of 10 different antemortem and postmortem factors on the expression levels of the individual protein species.

30       Multivariate analysis revealed that the quantities of 8 distinct spots were related

independently to one or more psychiatric diagnoses. Six (nos. 74, 76, 119, 141, 311, and 500) exhibited a decreased level in the brains of individuals with psychiatric disorders as compared to unaffected controls. Two proteins (nos. 182 and 226) displayed increased levels compared to controls (Table 1 and Figure 3). The actual measurements of the proteins are displayed in Figure 2. The univariate analysis does not correct for the antemortem and postmortem factors included in the multivariate analysis. Therefore the p values are different and usually higher than those reported in Table 1. The eight proteins were eluted from the 2-D gels, and tryptic peptides were sequenced by electrospray mass spectrometry. The sequenced peptides were identified against known proteins in protein databanks (Table 2).

Four of the protein species (76, 119, 311, and 500) that were decreased in one or more diseases, were identified as glial fibrillary acidic protein (GFAP). The four spots make up the majority of a 5-6 spot charge series visible on the two-dimensional gels (Figure 2, inset). The pI and MW values of the spots are depicted in comparison to the theoretical values of the GFAP in Table 3. The center spot, 189, had been identified previously as GFAP and the level thereof was not altered significantly in any of the disease states.

There were 4 additional protein species with diagnosis-related alterations in frontal cortex expression (Table 2). The core 1 protein of the

ubiquinone cytochrome C reductase complex was decreased significantly. Carbonic anhydrase I was increased significantly in the frontal cortex regions of individuals with depression. The  
5 pyrimidine-metabolizing enzyme dihydropyrimidinase-related protein 2 was decreased in individuals with schizophrenia, bipolar disorder and major depressive disorder. A single spot containing both fructose biphosphate aldolase C and  
10 aspartate amino transferase was increased in all three diseases.

### EXAMPLE 3: COMBINATIONS OF PROTEIN BRAIN MARKERS

Multivariant statistical analysis via principle  
15 component analysis (PCA) was performed on the same 89 brain samples. The patterns were calibrated by scaling on spots that were present in at least 80% of the gels to reduce variation due to loading or staining.

20 That approach can provide disease discrimination under circumstances where no single marker protein or gene can distinguish disease or treatment from the control group.

Spots were selected based on the student T-test  
25 significance without being stringent ( $p < 0.01$ ). Only spots showing a difference between controls and group 2 (schizophrenia), group 3 (bipolar disorder) or group 5 (depression), and present in at least 50% of the patterns in a group were selected.  
30 Differences that showed up due to group 4



(Huntington's) or group 6 (miscellaneous) were left aside. One pattern per group (except group 6) also was removed due to having scale factors above 1.9.

Of the 19 spots selected (Figures 5a, 5b and 5 6), two analyses were run: 1) with 19 spots and 2) with 19 spots + 30 spots previously chosen for PCA spots. The total number of spots is 37 in the second analysis.

A panel of the 19 proteins was found in which 10 coordinated abundance differences could distinguish between normal and depression samples.

The percentages of explanation of PC 1, PC 2 and PC 3 are very similar in both cases:

PC 1 + PC 2 = 31% (19 spots) and 28% (37 15 spots)

PC 1 + PC 2 + PC 3 = 41% (19 spots) and 37% (37 spots)

The reference values for random variation are: 5.3% per axis in the 19 analysis and 2.7% per axis 20 in the 37 analysis.

The analyses appear to show that increasing the number of patterns significantly increases the global variability and causes the selection of spots based on the techniques in Example 2 above.

25 In the 19 variable analysis, the variables contributing the most to the space PC 1-PC 2 are the spots 185, 62, 159, 402, 226, 152, and 191. That is consistent with the general observation that increasing the number of samples tends to increase 30 the global variability and to decrease the

significance of the individual spots associated to a principal component or plane.

In interpreting the data one may look at the structure of the space focusing on variation associated to specific individuals rather than groups, see Figure 4.

TABLE 4  
Analysis 19 spots Analysis 37 spots

MSN	PC 1 - PC 2	PC 1 - PC 3	PC 1 - PC 2	PC 1 - PC 3
61		X	X	
62	X	X	X	
74			X	
103			X	X
152	X		X	
159	X			X
185	X		X	
191	X			
195			X	X
226	X			
402	X		X	
512	X		X	X
621	X		X	X

10

The coefficients used to multiply by the density values to generate the data from Figure 4 are given in Figure 7.

It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplification of preferred embodiments. Those

skilled in the art will envision other modifications within the scope and spirit of the claims appended hereto.

All patents and references cited herein are  
5 incorporated explicitly by reference in their entirety.

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What is claimed is:

1. A method for diagnosing a brain disorder comprising;

obtaining a biological sample from a patient,  
measuring an amount of at least one protein,

nucleic acid encoding said protein, substrate  
utilized by said protein, or product produced by  
said protein in said biological sample, and

comparing the result to a standard amount found  
in standardized biological sample.

2. The method of claim 1 wherein the protein  
is selected from the group consisting of the  
proteins listed in Table 2.

3. The method of claim 1 wherein the protein  
is selected from the group consisting of the  
proteins listed in Table 4.

4. A method for determining whether a  
candidate drug is effective in treating a brain  
disorder comprising:

contacting the candidate drug with the brain  
cells of a subject,

obtaining a biological sample from said  
subject,

measuring an amount of at least one of the  
following proteins, nucleic acids encoding said  
proteins, substrates utilized by said protein, or

products produced by said proteins in said biological sample, and

comparing the result to standard amounts found in standardized biological samples.

5. The method of claim 1 wherein the protein is selected from the group consisting of the proteins listed in Table 2.

6. The method of claim 1 wherein the protein is selected from the group consisting of the proteins listed in Figure 7.

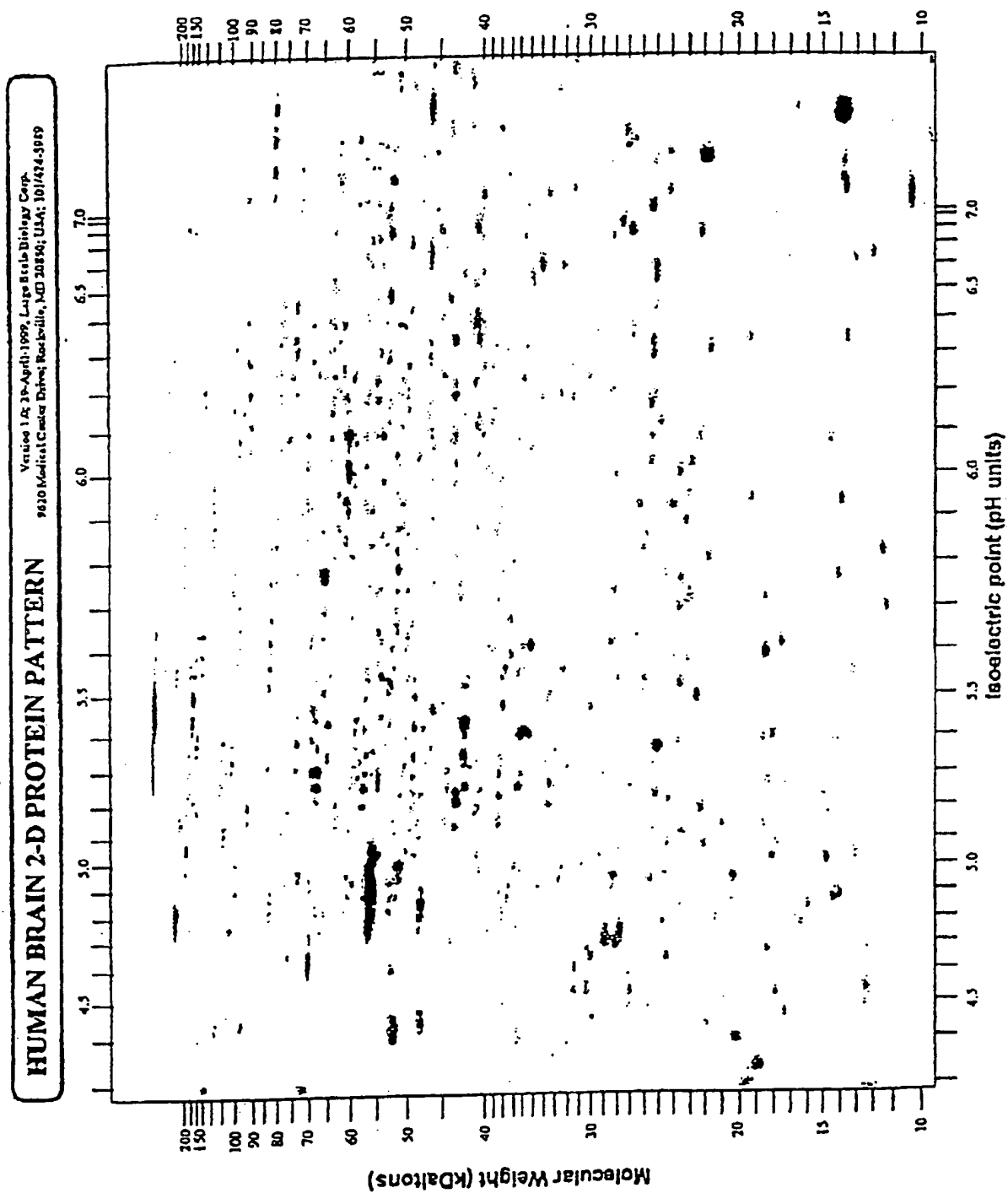
7 A pharmaceutical composition comprising an effective amount of a protein having abnormal levels in an individual with a brain disorder and a pharmaceutically acceptable carrier.

8. A pharmaceutical composition comprising an effective amount of a nucleic acid encoding a protein having abnormal levels in an individual with a brain disorder and a pharmaceutically acceptable carrier.

9. The pharmaceutical composition of claim 7 or 8 wherein the pharmaceutical composition is encapsulated in a liposome or micelle.

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FIGURE 1



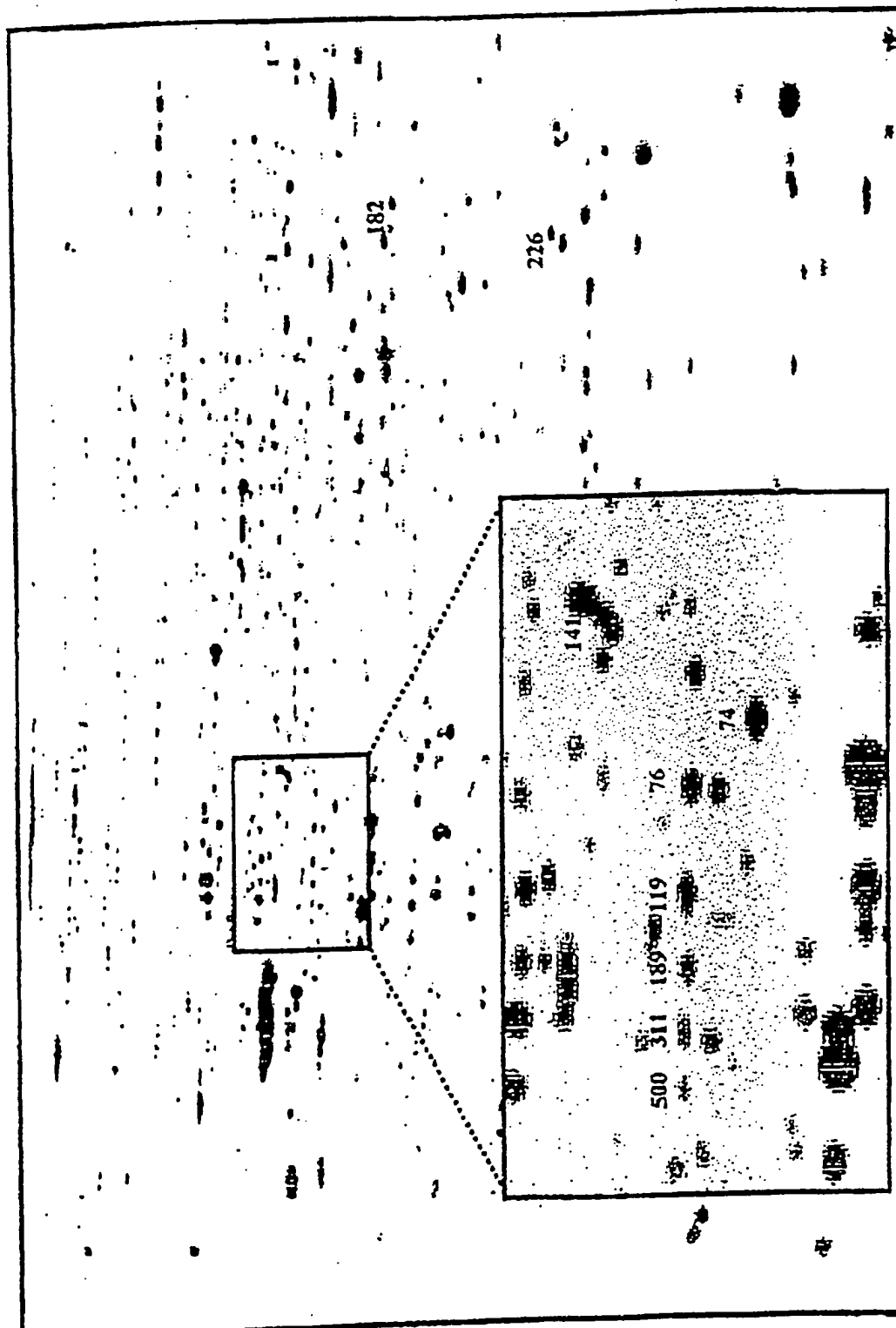


Figure 2. Spots showing disease related variations in levels. 74; ubiquinone cytochrome c reductase core complex protein I. 76, 119, 189, 311, and 500; glial fibrillary acidic protein. 141; dihydropyrimidinase related protein 2. 182; fructose biphosphate aldolase. 226; carbonic anhydrase I.

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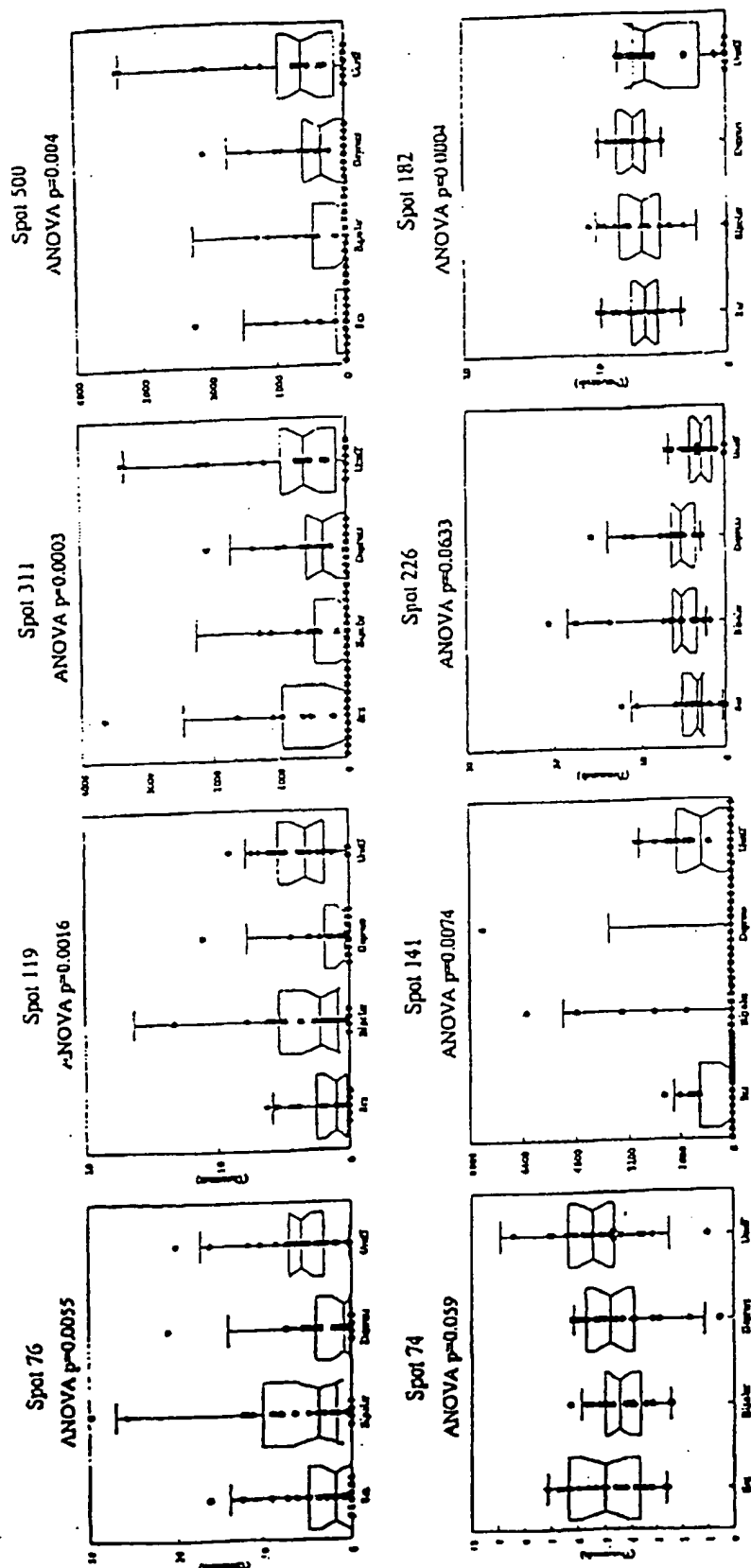


Figure 3. Box and whisker plots of protein levels for various diseases. Levels are measured in relative units as described in Materials and Methods. Boxes are drawn from the 25th to the 75th percentile of the data, with the median indicated by the line across the indentation. Error bars from the 5th to 95th percentile are also shown. ANOVA values measure the statistical differences between all groups, and were calculated on Statistica software.



PCA Comparison of Human Brain Protein Patterns

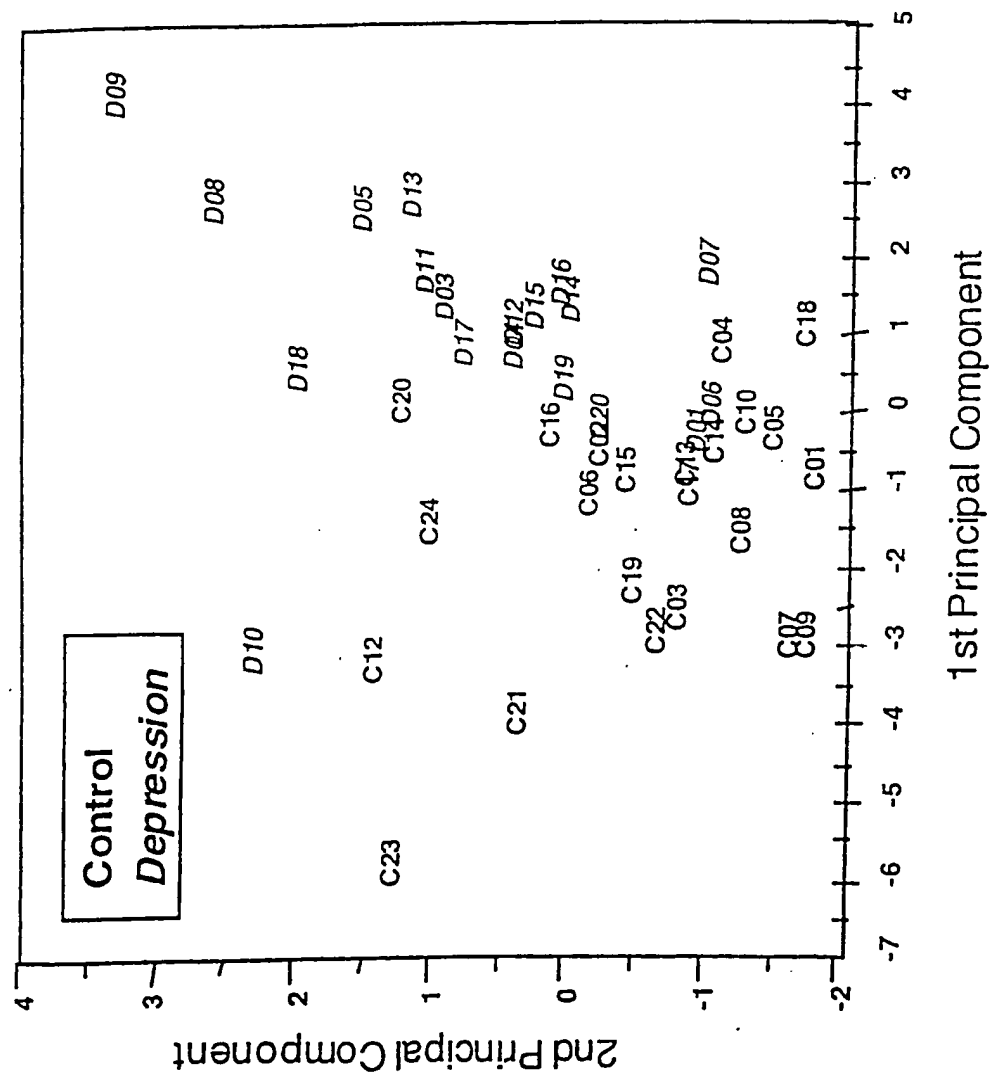


FIGURE 4

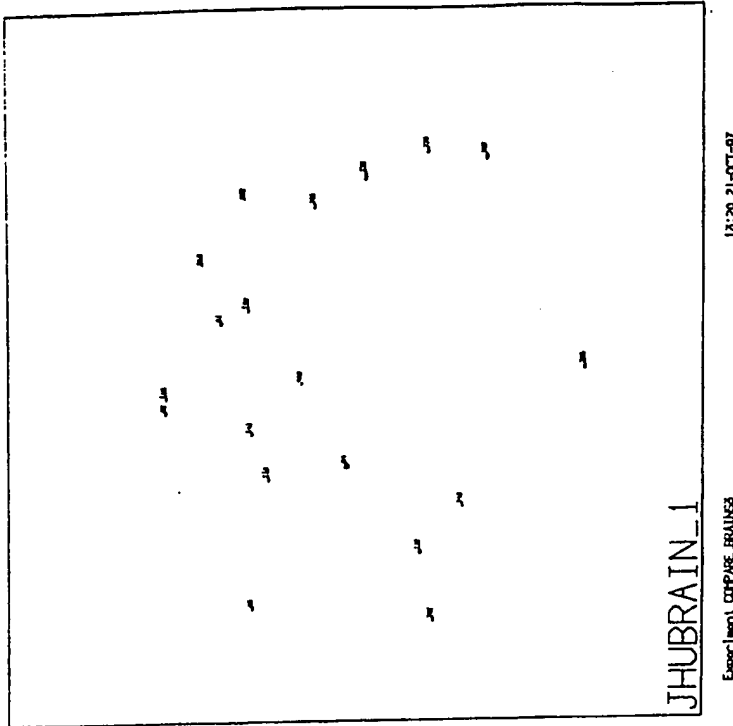


Figure 5B

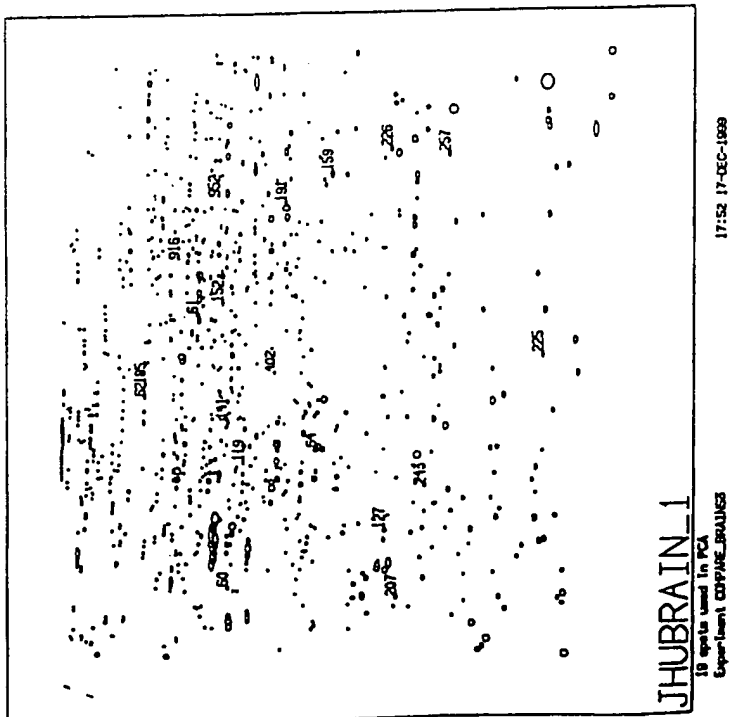
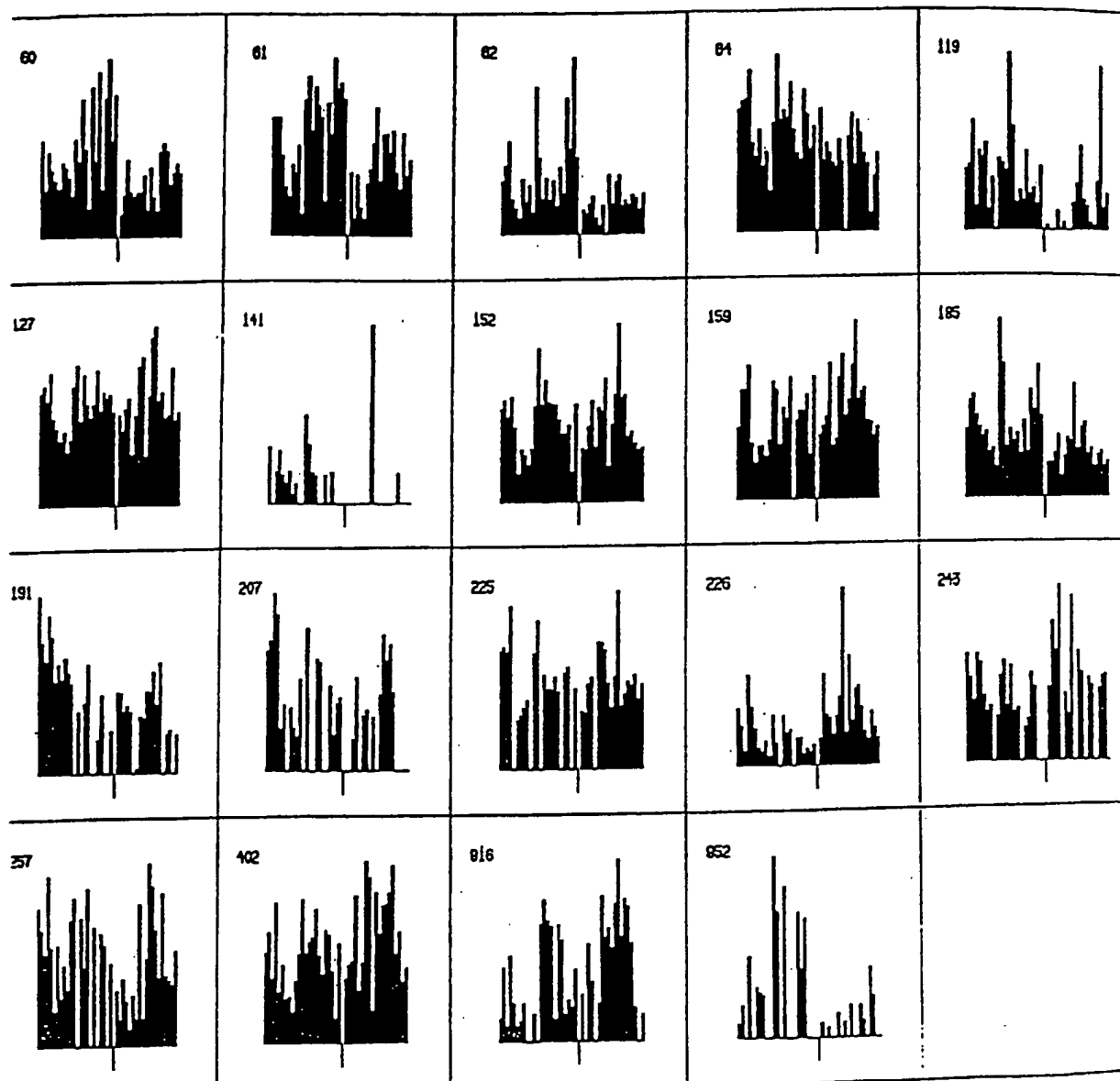


Figure 5A



19 spots used in PCA  
Experiment COMPARE\_BRAINS3

17:34 17-DEC-1999

Figure 6

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## Figure 7

Spot	PRIN1	PRIN2
S60	-0.211	-0.154
S61	-0.281	0.202
S62	-0.397	0.241
S64	-0.092	-0.254
S119	-0.214	0.226
S127	0.243	0.083
S141	0.089	0.143
S152	0.319	-0.208
S159	0.299	0.268
S185	-0.345	0.338
S191	-0.092	-0.432
S207	0.018	-0.077
S225	0.147	-0.095
S226	0.293	0.258
S243	0.166	0.235
S257	0.119	-0.260
S402	0.315	0.274
S916	0.119	0.197
S952	-0.127	0.023